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Method for the facile transformation of marine purple photosynthetic bacteria using chemically competent cells

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Abstract

Marine purple photosynthetic bacteria are ideal organisms for the production of useful materials at reduced costs and contributing to a sustainable society because they can utilize sunlight, seawater, and components of air, including carbon dioxide and nitrogen gases, for their growth. However, conjugation is the only applicable method for the transformation of marine purple photosynthetic bacteria so far. Here, we examined a calcium chloride-mediated method for the transformation of marine purple photosynthetic bacteria. Plasmid DNAs containing the kanamycin resistance gene were successfully transferred into chemically competent cells of two strains of marine purple photosynthetic bacteria (*Rhodovulum sulfidophilum* and *Roseospira marina*). Heat shock treatment increased the transformation efficiency in *R. sulfidophilum*, whereas the addition of cell-penetrating peptide did not improve it. We also found that prolonged incubation in agar plates containing kanamycin led to spontaneous mutation of the 16S rRNA, resulting in kanamycin resistance in *R. marina*. Thus, we developed an efficient and facile transformation method using chemically competent cells of marine purple photosynthetic bacteria with calcium chloride.

KEYWORDS
16S rRNA, calcium chloride method, chemically competent cells, marine purple photosynthetic bacteria, transformation

1 | INTRODUCTION

Transformation, which introduces foreign DNA into cells, is an essential technology for genetic engineering. A number of transformation methods have been established (Aune & Aachmann, 2010). In the case of bacteria, electroporation, conjugation, natural competence, and chemical competence methods have been used to transfer foreign DNA into the cells. Electroporation is a physical method that temporally permeabilizes the cell membranes by applying a short electrical pulse. Conjugation is the transfer of DNA between donor and recipient cells via direct cell-to-cell contact. Natural competence is the ability to uptake DNA from the environment and has been utilized for transformation in many bacterial species (Lorenz & Wackernagel, 1994). Competency is known to be induced by chemical treatment, such as treatment with dimethyl sulfoxide (DMSO) (Hanahan, 1983), divalent cations (Dagert & Ehrlich, 1979a; Mandel & Higa, 1970), or polyethylene glycol (PEG)(Klebe, Harriss, Sharp, & Douglas, 1983). Applicable and efficient transformation methods have been selected according to the physiological and biological properties of bacterial species.

The use of photosynthetic organisms to produce value-added materials and chemicals is one of the potential methods to reduce costs and can contribute to a sustainable social system because these organisms can utilize sunlight and carbon dioxide (CO_2) in the

air for their growth and producing activity. Some species of purple photosynthetic bacteria are known to fix nitrogen in addition to CO_2 (Gallon, 2001; McKinlay & Harwood, 2010). This means that they can use nitrogen gas (N_2) and CO_2 in the air as nitrogen and carbon sources for their growth, respectively. Marine bacteria have other advantages as host organisms of biological production because high-salinity culture medium can reduce the risk of biological contamination, and the highly abundant seawater can be used as a growth medium. We focused on marine purple photosynthetic bacteria as host microorganisms for the production of polyhydroxyalkanoates, which are a family of biopolyesters (Foong, Higuchi-Takeuchi, & Numata, 2019; Higuchi-Takeuchi, Morisaki, Toyooka, & Numata, 2016; Higuchi-Takeuchi & Numata, 2019). However, genetic transformation technologies, such as the use of high-expression promoters and transformation methods for marine purple photosynthetic bacteria, have not been well established to date.

Although conjugation methods are widely used for transformation in purple photosynthetic bacteria, the protocol for conjugation contains many steps and is time-consuming. Moreover, there are a limited number of vectors for the conjugation method. Another way of transformation in purple photosynthetic bacteria is the electroporation method. However, high-salt medium, which is necessary for marine bacteria, reduces the electroporation efficiency. Therefore, only conjugation is an applicable method of transformation for marine purple photosynthetic bacteria.

In this study, we examined using the chemical competence method by $CaCl_2$ treatment for the transformation of marine purple photosynthetic bacteria. As a result, plasmid DNA (pDNA) has been successfully introduced into two marine purple photosynthetic bacterial strains. Heat shock treatment was effective in the transformation of $CaCl_2$ competent cells, while cell-penetrating peptide (CPP) did not enhance the efficiency. We also found that prolonged incubation for more than 30 days led to mutation of the 16S rRNA gene, resulting in kanamycin resistance.

2 | MATERIALS AND METHODS

2.1 | Culture conditions

The marine purple photosynthetic bacteria investigated in this study were obtained from biological resource centers (RIKEN BioResource Center and ATCC). Marine purple photosynthetic bacteria were cultured in Marine Broth, Marine Agar 2,216 (Difco, Detroit, USA), and JCM 568 medium (https://www.jcm.riken.jp/cgi-bin/jcm/jcm_grmd?GRMD=568), the last of which contains the following components per liter: KH_2PO_4 0.5 g, $CaCl_2.2H_2O$ 0.15 g, $MgSO_4$ $0.7H_2O$ 1.5 g, NH_4Cl 0.6 g, NaCl 20 g, sodium pyruvate 3.0 g, yeast extract 0.4 g, ferric citrate 0.25 mg, $ZnCl_2.5H_2O$ 70 μg, $MnCl_2.4H_2O$ 100 μg, H_3BO_3 60 μg, $CoCl_2.6H_2O$ 200 μg, $CuCl_2.2H_2O$ 20 μg, $NiCl_2.6H_2O$ 20 μg, $Na_2MoO_4{\cdot}H_2O$ 40 μg, vitamin B_{12} 2 mg, 0.1% $NaHCO_3$, and 2 mM $Na_2S_2O_3$. The pH was adjusted to 6.8 with NaOH. Marine Broth and Marine Agar 2,216 were used for the growth of *Rhodovulum sulfidophilum* and *Roseospira marina*. The 568 medium was used as the growth medium of *Roseospira visakhapatnamensis* and *Roseospira goensis* cells. Marine purple photosynthetic bacteria were grown under continuous far-red LED light (730 nm, VBP-L24-C3, Valore, Tokyo, Japan) at 30°C in plastic tubes filled with medium to the tops of the necks.

2.2 | Preparation of chemically competent cells

Cells were harvested in the log phase (OD_{660} = approximately 2.0), diluted with growth media to OD_{660} = 0.1, and cultured for approximately 20 hr (OD_{660} = approximately 0.6 to 0.8). Cells were transferred to a 50-mL plastic tube and incubated on ice for 10 min. Cooled cell cultures were centrifuged at 3,000 rpm without braking at 0°C for 5 min, and the supernatant was discarded. Cells were suspended in 8 ml of cooled 50 mM $CaCl_2$ and centrifuged at 2,500 rpm without braking at 0°C for 3 min. The supernatant was discarded, and cells were suspended in 8 ml of cooled 50 mM $CaCl_2$. The resulting cell suspensions were incubated on ice for more than 30 min and centrifuged at 2,500 rpm without braking at 0°C for 3 min. The supernatant was discarded, and finally, the cells were suspended in 4 ml of cooled 50 mM $CaCl_2$/ 15% glycerol and incubated on ice for more than 2 hr. Then, 100 μl of chemically competent cells were transferred into 1.5-mL tubes and frozen in liquid nitrogen. Chemically competent cells were kept at −80°C until use.

2.3 | Preparation of peptide–pDNA complex and transformation

Plasmid (pBBR1MCS-2)(Kovach et al., 1995) carrying the kanamycin-resistant gene *npt II* was prepared using a QIAprep Spin Miniprep Kit (QIAGEN, Dusseldorf, Germany) according to the standard protocol. When we used a low-purity plasmid with a 260/280 ratio of ~ 1.7, the transformation efficiency was low compared to that with a highly pure plasmid (260/280 ratio of approximately 1.9). Then, 1 μg of pDNA was mixed with peptides $(BP100)_2K_8$: KKLFKKILKYLKKLFKKILKYLKKKKKKKK at an N/P ratio of 0.1 and incubated at room temperature for 10 min to form peptide–pDNA complexes (Lakshmanan, Kodama, Yoshizumi, Sudesh, & Numata, 2013). Competent cells were thawed on ice and mixed with 1 μg of pDNA or peptide–pDNA complexes. Cells were incubated for more than 30 min on ice. For heat treatment, cells were incubated for 1 min at 42°C followed by incubation on ice for 2 min. After incubation on ice, 1.5 ml of growth medium without antibiotics was added to cells, and cells were preincubated at 30°C overnight under far-red illumination. After preculture, cell cultures were spread in Marine Agar 2,216 (Difco, Detroit, USA) or 568-medium agar plates containing 100 mg/L kanamycin. Cells were cultured at 30°C under far-red light for 14 days (short-term incubation) and up to 37 days (long-term incubation). For the long-term incubation study, transformations of *R. marina* were carried out two times under four transformation conditions (with or without CPP and heat shock treatment). Eight kanamycin-resistant colonies were examined after 20 days of incubation

(long-term incubation) for sequencing analysis of 16S ribosomal RNA (16S rRNA).

2.4 | RT-PCR analysis

Total RNA was extracted using the RNeasy Mini Kit (QIAGEN, Dusseldorf, Germany) according to the manufacturer's protocol. Using 0.1 μg of RNA as a template, cDNA was synthesized by a QuantiTect Reverse Transcription Kit (QIAGEN, Dusseldorf, Germany) following the manufacturer's protocol. To determine the expression levels of the *npt II* gene, the following sets of primers were used: npt-*F* (5'- AGA CAA TCG GCT GCT CTG AT −3') and npt-R (5'- CTC GTC CTG CAG TTC ATT CA −3'). Primer mix from the bacterial 16S TaKaRa rDNA PCR Kit (TaKaRa, Shiga, Japan) was used for amplification of the 16S rRNA gene.

2.5 | Southern blotting analysis

Genomic DNA was isolated from *R. marina* cells using the CTAB method (C. N. Jr. Stewart & L. E. Via, 1993). Approximately 10 μg of genomic DNA was digested with BamHI, run on a 1% agarose gel, and transferred to a Hybond N + nylon membrane (GE Healthcare, Chicago, IL, USA). The *GAPDH* gene was used as an internal reference gene. Probe preparation for *GAPDH* and *npt II* genes and detection were performed using the CDP-Star AlkPhos Direct Labeling and Detection system (GE Healthcare, Chicago, IL, US) according to the manufacturer's protocol. The following primer sets were used for probe preparation: for *GAPDH*, GAPDH-Fw (5'- CAA GGT AGC AAT CAA CGG CT −3') and GAPDH-Re (5'- TGT GGA TCG TGG TCA TGA AG −3'); for *npt II*, nptR-F2 (5'- TGC TCC TGC CGA GAA AGT AT −3') and npt-R2 (5'- AGA ACT CGT CAA GAA GGC GA −3'). *R. sulfidophilum* genomic DNA and pBBR1MCS-2 were used for PCR templates.

2.6 | Sequence analysis of the 16S rRNA

Genomic DNA of *R. marina* cells was extracted using DNase Blood and Tissue Kit (QIAGEN, Dusseldorf, Germany) according to the manufacturer's protocol. A bacterial 16S TaKaRa rDNA PCR Kit (TaKaRa, Shiga, Japan) was used for amplification of the 16S rRNA and sequencing.

3 | RESULTS AND DISCUSSION

3.1 | Antibiotic resistance of marine purple photosynthetic bacteria

To develop a transformation method for marine purple photosynthetic bacteria, the plasmid pBBR1MCS-2 (Kovach et al., 1995), carrying the kanamycin-resistant gene *npt II*, was selected in this study. pBBR1MCS is a broad-host range plasmid and was used in some previous studies of purple photosynthetic bacteria (Garcia-Contreras, Celis, & Romero, 2004; Pinta, Picaud, Reiss-Husson, & Astier, 2002). We examined the kanamycin sensitivity of several strains of marine purple photosynthetic bacteria. As a result, four strains (*R. sulfidophilum*, *R. marina*, *R. visakhapatnamensis*, and *R. goensis*) exhibited resistance to more than 100 mg/L kanamycin (Figure A1). These strains were examined for transformation using kanamycin resistance as a selectable marker.

First, we tried a natural transformation method using *R. sulfidophilum*, which is a representative strain of marine purple photosynthetic bacteria. pDNA was mixed with cell cultures, and cells were plated on agar plates containing kanamycin. We examined several conditions, such as high pDNA content and various concentrations of cell cultures. However, the natural transformation method was not successful in *R. sulfidophilum*.

The natural transformation method was also investigated using the CPP-based technique. CPPs are composed of 5–30 amino acids and have the ability to penetrate the cells. We developed a peptide-based gene delivery system using fusion peptides consisting of CPP and lysine- and histidine-rich polycationic peptides. In this system, negatively charged plasmid DNA preferentially interacts with polycationic peptides and condenses through electrostatic interactions, and the CPP region functions as a cell-penetrating motif. This fusion peptide carrier was applied to DNA, RNA, and protein delivery into plants, tobacco BY-2 cells, and human cells (Lakshmanan et al., 2013; Ng et al., 2016; Numata, Ohtani, Yoshizumi, Demura, & Kodama, 2014). BP100 (KKLFKKILKYL) was originally designed as an antimicrobial peptide (Badosa et al., 2007) and shown to have cell penetration ability in tobacco BY-2 cells (Eggenberger, Mink, Wadhwani, Ulrich, & Nick, 2011). Previously, we compared the penetration efficiency of BP100 monomer and dimer in *E. coli* and demonstrated that the dimer of BP100 exhibited higher penetration efficiency than the monomer (Oikawa, Islam, Horii, Yoshizumi, & Numata, 2018). Moreover, $(BP100)_2K_8$ showed better protein delivery into the cells of plant leaves than $BP100(KH)_9$ (Ng et al., 2016). Therefore, we evaluated the effect of $(BP100)_2K_8$ on the natural transformation of *R. sulfidophilum* cells.

Addition of more than 10 g/L CPP led to severe growth inhibition of *R. sulfidophilum* (Figure A2). However, the CPP-pDNA complex in which CPP was mixed with plasmid DNA in solution did not affect the growth of *R. sulfidophilum* cells. The CPP-pDNA complex prepared at an N/P ratio of 0.1 was shown to be effective for transformation in *Escherichia coli* in a previous study (Islam et al., 2019). The CPP-pDNA complex was prepared with an N/P ratio of 0.1 using $(BP100)_2K_8$ and pBBR1MCS-2 and mixed with *R. sulfidophilum* cell cultures. These cell cultures containing peptide–pDNA complexes were selected on plates containing kanamycin after preincubation. However, transformants could not be obtained despite repeated experiments. We previously demonstrated that the optimal CPP was variable among the plant species using a library containing 55 types of CPP (Numata et al., 2018). Although $(BP100)_2K_8$ is one of the best CPPs against *E. coli*, the CPP did not function with *R. sulfidophilum* in this study. CPPs other than $(BP100)_2K_8$ might be effective for marine purple photosynthetic bacteria.

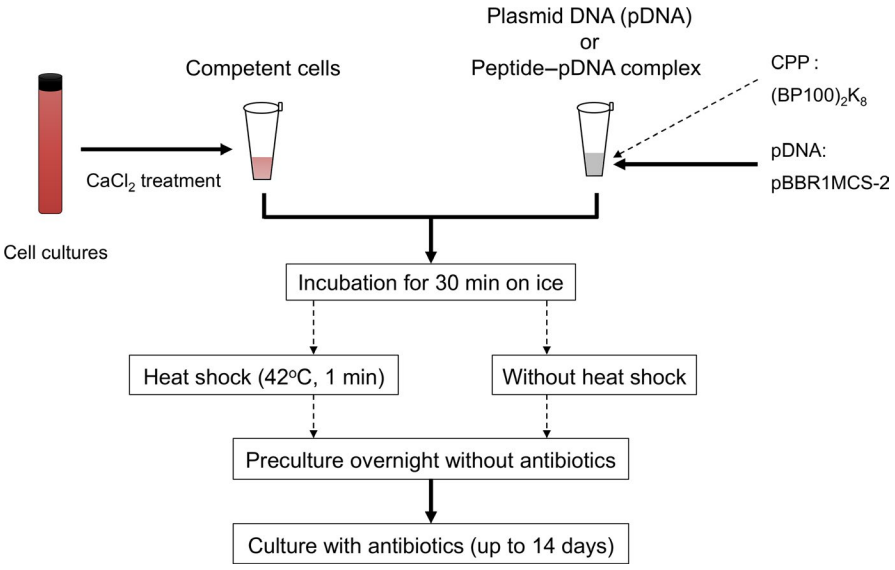

FIGURE 1 Scheme for the transformation of marine purple photosynthetic bacteria. Chemically competent cells were prepared by the $CaCl_2$ method. Plasmid DNA (pBBR1MCS-2) was mixed with competent cells in the presence or absence of CPP ($(BP100)_2K_8$). After incubation on ice for 30 min, competent cells were treated with or without heat shock. Four transformation conditions (with or without CPP and heat shock treatment) were examined. Common (solid lines) and alternative (dashed lines) procedures are shown in arrows

3.2 | $CaCl_2$-induced transformation

Next, we examined calcium chloride-induced transformation in *R. sulfidophilum*. Calcium chloride treatment is widely used for the transformation of *E. coli* (Dagert & Ehrlich, 1979b; Weston, Brown, Perkins, Saunders, & Humphreys, 1981) and other bacteria, including purple photosynthesis bacteria (Fornari & Kaplan, 1982; Russo, Panangala, Wood, & Klesius, 2009). The transformation procedure is summarized in Figure 1. Chemically competent cells were prepared using *R. sulfidophilum* cells by calcium chloride treatment. Chemically competent cells were mixed with plasmid DNA and preincubated overnight without kanamycin followed by incubation with kanamycin. Kanamycin-resistant colonies appeared after approximately 7–14 days of incubation, as shown in Figure 2a.

Heat shock treatment is commonly used in $CaCl_2$-mediated transformation (Mandel & Higa, 1970). We examined the effects of heat shock treatment at 42°C for 1 min and the CPP-pDNA complex on the transformation efficiency of *R. sulfidophilum* (Figure 2b).

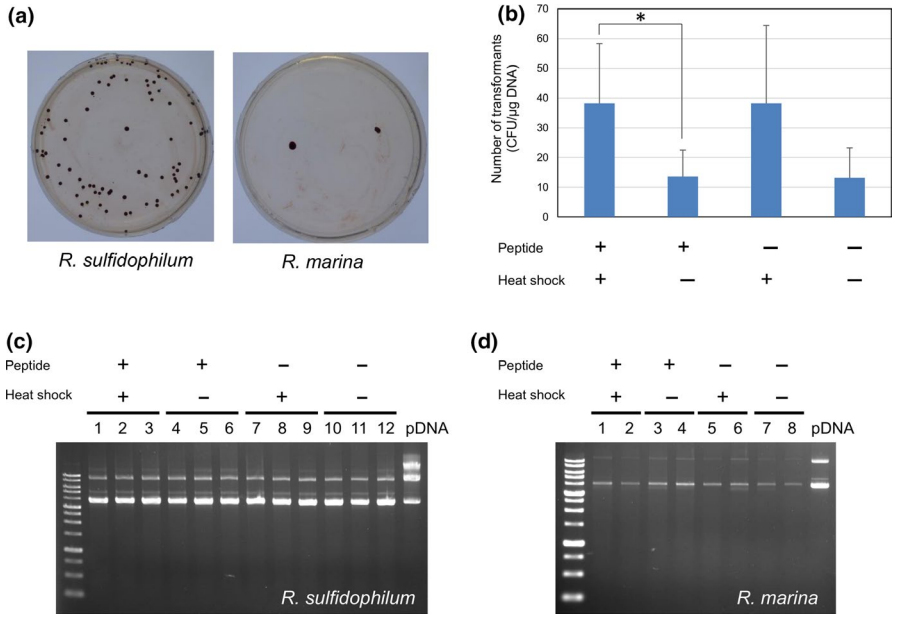

FIGURE 2 Transformation of marine purple photosynthetic bacteria. (a) Kanamycin-resistant colonies of *R. sulfidophilum* and *R. marina*. Photographs were taken after 14 days of incubation. Transformation was carried out by heat shock treatment in the presence of peptide. (b) Effects of CPP and heat shock on the transformation efficiency. Transformations were carried out in the presence or absence of CPP and heat shock treatment. The number of kanamycin-resistant colonies was counted after 14 days of incubation. The asterisk indicates a significant difference ($p < .05$). Plasmid extraction from kanamycin-resistant colonies of *R. sulfidophilum* (c) and *R. marina* (d). Plasmid solutions extracted from kanamycin-resistant colonies were analyzed by 1% agarose gel. Transformations were carried out in the presence or absence of CPP and heat shock treatment. Three kanamycin-resistant colonies (*R. sulfidophilum*) and two colonies (*R. marina*) from each set of conditions were analyzed in agarose gels

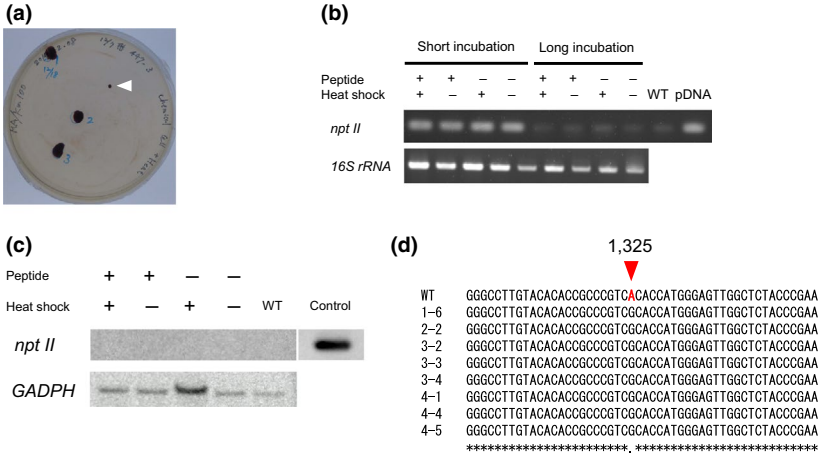

FIGURE 3 Kanamycin-resistant *R. marina* cells after long-term incubation. (a) Kanamycin-resistant colonies of *R. marina* after 30 days of incubation. Kanamycin-resistant colonies appeared after 30 days of incubation, and they are shown in white arrows. (b) RT-PCR analysis of the *npt II* gene. Transformations were carried out in the presence or absence of CPP and heat shock treatment. RNA was extracted from short-term and long-term incubated kanamycin-resistant colonies. RT-PCR was carried out using the *npt II* gene-specific primer and the 16S rRNA gene (internal standard)-specific primer sets. (c) Southern blotting analysis of the *npt II* gene. Transformations were carried out in the presence or absence of CPP and heat shock treatment. Genomic DNA was extracted from long-term incubated kanamycin-resistant colonies of *R. marina*. Southern blotting analysis was carried out using *npt II*- and the 16S rRNA gene (internal standard)-specific probes. The PCR product of the *npt II* gene was used as a positive control. (d) Alignment of the 16S rRNA sequences between WT and long-term incubated kanamycin-resistant colonies of *R. marina*. 16S rRNA sequences of WT and eight long-term incubated kanamycin-resistant colonies of *R. marina* were determined. Sequences show a 3' part of the 16S rRNA. The red triangle shows position 1,325 of the 16S rRNA

The number of transformants increased significantly by heat shock treatment in the presence and absence of CPP. Thus, the transformation efficiency was not improved by the addition of CPP but by the heat shock treatment. Transformation was also performed using another plasmid, pJRD215, which has a broad-host range (Davison, Heusterspreute, Chevalier, Hathi, & Brunel, 1987). The size of pJRD215 is 10.2 kb, and it is larger than pBBR1MCS-2 (5.1 kb). Kanamycin-resistant colonies could be obtained by heat shock treatment after 14 days of incubation. Colony-forming units (CFU) per μg DNA were 1.8 ± 1.5 and 3.4 ± 4.4 (n = 5) in the presence or absence of CPP, respectively. A large plasmid size could be introduced into *R. sulfidophilum* cells in our method, although the transformation efficiency of pJRD215 was low compared to that of pBBR1MCS-2.

Chemically competent cells of another marine purple photosynthetic bacteria, *R. marina*, were prepared and investigated for transformation efficiency using pBBR1MCS-2. As a result, only several kanamycin-resistant colonies were observed for *R. marina* (Figure 2a, Table A1). The presence of plasmid was confirmed by extraction of plasmid from kanamycin-resistant cells of *R. sulfidophilum* (Figure 2c) and *R. marina* (Figure 2d), indicating that pDNAs were successfully transferred into the cells.

In contrast to *R. sulfidophilum* and *R. marina*, no kanamycin-resistant colony was found in *R. visakhapatnamensis* and *R. goensis* under all conditions (Table A1). Both *R. visakhapatnamensis* and *R. goensis* showed poor growth compared to *R. sulfidophilum* and *R. marina*. In these experiments, we found that the purity of pDNA is important for efficient transformation. Further optimization of various parameters, such as growth conditions and cell culture volume, is required to achieve transformation.

3.3 | Long-term incubation

Time-dependent changes in the number of kanamycin-resistant colonies of *R. marina* are shown in Table A2. When the incubation period with kanamycin was extended to more than 20 days during long incubation times, additional kanamycin-resistant colonies appeared (Table A2, white arrow in Figure 3a). An average of 1.0 colony per plate was observed from 8 agar plates during long-term incubation. Plasmid extraction from kanamycin-resistant cells was carried out after a long period of incubation. However, no plasmid could be extracted and detected by agarose gel electrophoresis. Gene expression of *npt II* was analyzed by RT-PCR analysis (Figure 3b). RT-PCR products were observed in short-term cultured kanamycin-resistant cells. On the other hand, *npt II* expression was hardly detected or quite low in long-term cultured cells. To check the possibility that the *npt II* gene was integrated into the genome, Southern blotting analysis was carried out using the *npt II* gene probe (Figure 3c). A hybridization signal was not observed in long-term cultured cells, suggesting that the *npt II* gene was not integrated into the genome.

It has been reported that mutation of 16S rRNA conferred kanamycin resistance in *E. coli* and *Mycobacterium tuberculosis* (Apirion & Schlessinger, 1968; De Stasio, Moazed, Noller, & Dahlberg, 1989; Suzuki et al., 1998). The sequences of the 16S rRNA in *R. marina* long-term incubated kanamycin-resistant cells were determined and compared to those of WT (Figure 3d). An A-to-G mutation was found at position 1,325 among all kanamycin-resistant *R. marina* cells. Mutations at position 1,408 in *E. coli* and 1,389 in *M. tuberculosis* lead to kanamycin and other antibiotic resistance (De Stasio et al., 1989; Suzuki et al., 1998), and this position is reported to be an important

region of antibiotic binding (Fourmy, Recht, Blanchard, & Puglisi, 1996). Multiple sequence alignment revealed that the mutations at position 1,408 in *E. coli* and 1,389 in *M. tuberculosis* corresponded to position 1,325 in *R. marina*. These results suggest that spontaneous mutation of the 16S rRNA occurred during a long incubation period of more than 30 days to lead to kanamycin resistance in *R. marina*. Thus, our observations demonstrated that long-term incubation with antibiotics for transformation should be avoided due to spontaneous mutation.

4 | CONCLUSIONS

Marine purple photosynthetic bacteria have the potential to be biological production systems contributing to the sustainable development of society. However, biotechnological tools have not been well established. In this study, we examined a transformation method for marine purple photosynthetic bacteria using competent cells treated with $CaCl_2$. pDNA with a kanamycin resistance gene was successfully transferred into two strains of marine purple photosynthetic bacteria. Heat shock treatment improved the transformation efficiency of competent cells of *R. sulfidophilum*. Long-term incubation with kanamycin led to spontaneous mutation of the 16S rRNA in *R. marina*, resulting in kanamycin resistance. Chemically competent cells can be stored at −80°C and used for transformation at any time. Thus, our method developed here generates ready-to-use competent cells of marine purple photosynthetic bacteria, which will expand the science and technology applications involving marine purple photosynthetic bacteria.

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CONFLICT OF INTERESTS

None declared.

AUTHOR CONTRIBUTIONS

M.H.-T. and K. N. conceived and designed the study. K.M. performed the experiments. M.H.-T. analyzed and interpreted the data and prepared the manuscript. M.H.-T. and K.N. approved the manuscript.

DATA AVAILABILITY STATEMENT

All data are provided in full in the results section of this paper and available from the corresponding author on reasonable request.

ETHICS APPROVAL

None required.

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APPENDIX A

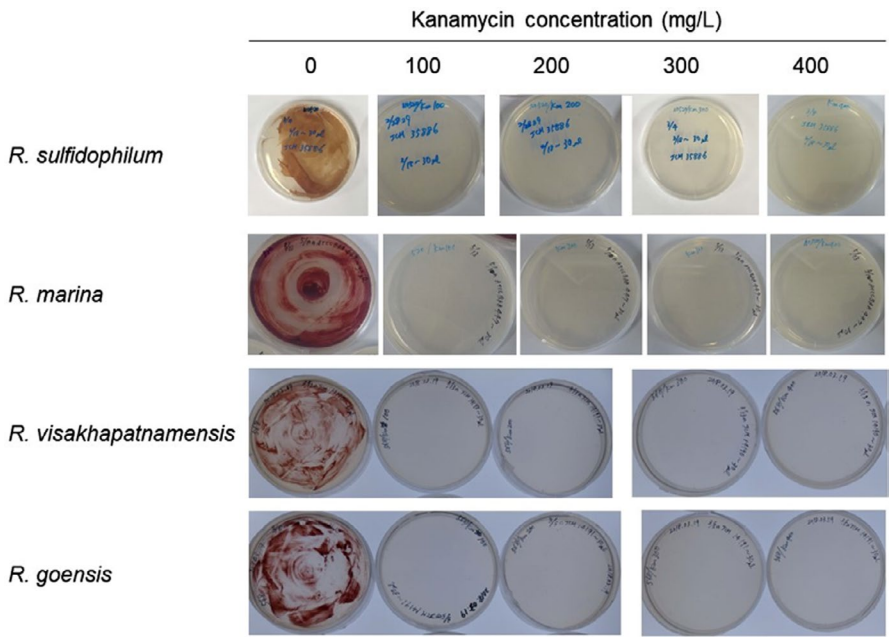

FIGURE A1 Kanamycin resistance tests of marine purple photosynthetic bacteria. Four strains of marine purple photosynthetic bacteria (*R. sulfidophilum*, *R. marina*, *R. visakhapatnamensis* and *R. goensis*) cells were cultured in the absence or the presence of 100, 200, 300 and 400 mg/L of kanamycin

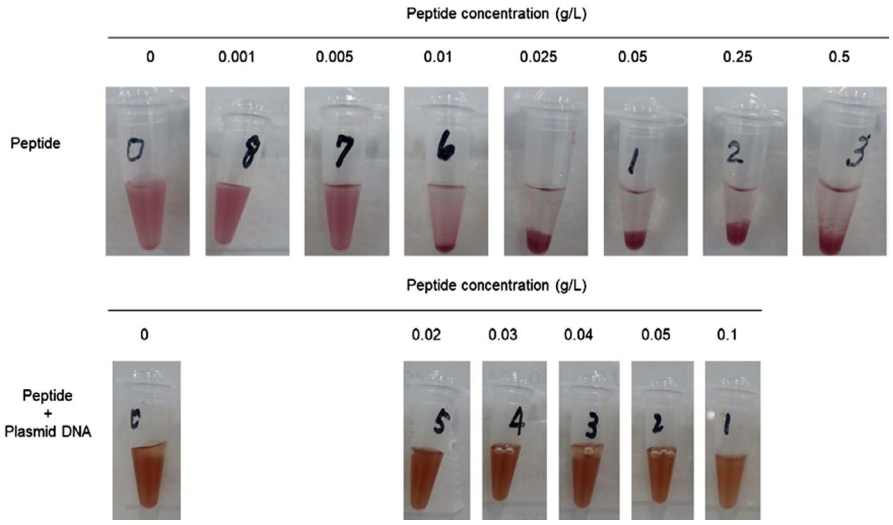

FIGURE A2 Cytotoxic effects of CPP peptide and peptide-pDNA complex. Various concentrations of CPP peptide ($(BP100)_2K_8$) ranging from 0.001 to 0.5 g/L were added to *R. sulfidophilum* cell cultures and incubated for 30 min at room temperature. The 1 μg of plasmid DNA was mixed with different concentrations of CPP peptides (0, 0.02, 0.03, 0.04, 0.05 and 0.1 g/L) and incubated at room temperature for 10 min. The peptide-pDNA complexes were added to *R. sulfidophilum* cell cultures and incubated for 30 min at room temperature

Peptide	+	+	-	-
Heat shock	+	-	+	-
R. marina	1.1 ± 1.9	1.1 ± 1.3	1.7 ± 1.1	0.7 ± 1.1
R. visakhapatnamensis	0	0	0	0
R. goensis	0	0	0	0

TABLE A1 Transformation efficiencies (CFU/μg DNA) of marine purple photosynthetic bacteria

Note: Four transformation conditions (with or without CPP peptide and heat shock treatment) were examined using pBBR1MSC-2 plasmid in three strains (*R. marina*, *R. visakhapatnamensis* and *R. goensis*). Transformation was carried out in the presence (+) or absence (-) of CPP peptide. After incubation on ice, cells were treated with (+) or without (-) heat shock.

TABLE A2 Time course changes in number of kanamycin resistant colonies of *R. marina*

(a) 1st transformation						
Time of incubation (days)	**6**	**10**	**20**	**27**	**38**	**Total**
+CPP, +Heat shock	4	1	0	1 (1–6)	0	6
+CPP	1	0	1 (2–2)	0	0	2
+Heat shock	1	0	1 (3–2)	0	1 (3–3)	3
DNA only	0	0	0	0	1 (4–1)	1

(b) 2nd transformation				
Time of incubation (days)	**6**	**11**	**27**	**Total**
+CPP, +Heat shock	0	2	0	2
+CPP	2	2	0	4
+Heat shock	3	0	1 (3–4)	4
DNA only	2	1	2 (4–4,4–5)	5

Note: Transformations of *R. marina* were carried out two times under four transformation conditions (with or without CPP peptide and heat shock treatment). Eight kanamycin resistant colonies found after 20-days of incubation (long-term incubation) shown in bold were examined for sequencing analysis of 16S rRNA. The number in parenthesis correspond to the 16S rRNA sequences in Figure 3d.